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Summary

Peripheral nerve injury is a common casualty and although peripheral nerve fibers retain a considerable regeneration potential also in the adult, recovery is usually rather poor, especially in case of large nerve defects. The aim of this paper is to address the perspectives in regeneration and tissue engineering after peripheral nerve injury by reviewing the relevant experimental studies in animal models. After a brief overview of the morphological changes related to peripheral nerve injury and regeneration, the paper will address the evolution of peripheral nerve tissue engineering with special focus on transplantation strategies, from organs and tissues to cells and genes, that can be carried out, particularly in case of severe nerve lesions with substance loss. Finally, the need for integrated research which goes beyond therapeutic strategies based on single approaches is emphasized, and the importance of bringing together the various complimentary disciplines which can contribute to the definition of effective new strategies for regenerating the injured peripheral nerve is outlined.

1. Introduction

Peripheral nerves are the organs which constitute the main part of the peripheral nervous system. They are made by fascicles of myelinated and unmyelinated nerve fibers (i.e. axons surrounded by glial ensheathings which represent the parenchyma of the organ) and by a complex stromal connective scaffold (Geuna et al., 2009). Peripheral nerves can also be named as nerves only since nerve fiber fascicles in the central nervous system are referred to as white matter (and not central nerves). By contrast, the term nerve fiber is used to refer both to central and peripheral axo-glial com-plexes and thus the type of nerve fiber must be always specified (either central or peripheral).

Nerves constitute a very rich web throughout the body and con-nect the central nervous system and the sensory and autonomic ganglia to the peripheral target organs of the motor and sensory pathways (Williams, 1999). The large extension of these organs makes them potentially affected by traumas in any site of the body. Peripheral nerve lesions are much more frequent than spinal cord lesions and their high incidence is at the basis of the continuously increasing interest of both basic and clinical researchers to the study on nerve repair and regeneration (Evans, 2001; Ruijs et al., 2005; Siemionow and Brzezicki, 2009). Nerve lesions do not usu-ally represent a threat for the patient's life but almost always affect the patient's quality of life which represents one of the main health targets of today's medicine (Battiston et al., 2009b).

In comparison to the central nervous system, nerve fibers in the peripheral nervous system retain, also in adulthood, a higher posttraumatic regeneration potential. However, in most cases, the clinical outcome after peripheral nerve lesions is far from being sat-isfactory and almost never functional recovery is complete (Höke, 2006; Lundborg, 2002; Midha, 2006; Millesi, 2006; Ruijs et al., 2005; Samii et al., 1997; Casha et al., 2008; Battiston et al., 2009b). There is thus a need for more research in nerve repair and regen-eration which brings together different disciplines which might contribute, not only to increase our knowledge about the biological mechanisms that underlie the complex sequence of events which follows nerve injury, but also to define the best strategies for opti-mizing posttraumatic nerve regeneration and, eventually, the full recovery of the patient's motor and sensory function (Siemionow and Brzezicki, 2009).

In the present review, we will first briefly outline the sequence of morphological events which follow a nerve lesion and then we will overview some of the most promising strategies that are cur-rently being

explored in experimental animal models to improve posttraumatic nerve regeneration.

2. Nerve injury and regeneration

The most popular classification of nerve injuries is still undoubt-fully the Sunderland's scale which includes five degrees which correspond to an increasing severeness of the lesion (Sunderland, 1951). The first-degree (also called neuropraxia) refers to injuries (usually compressions) that cause a block in the action potential conduction without loosing axonal continuity. In second-degree injuries, axonal continuity is lost without damage of the surround-ing glial and connective structures; thus, axonal regrowth and its proper orientation is optimal being guided by the original glial tubules in the distal nerve stump. In third-degree injuries, also the endoneurial structures are disrupted and thus, although nerve continuity is maintained, regrowth of damaged axons and espe-cially their orientation to the proper target can be poorer than in second-degree lesions. Sundenrland's four-degree refers to nerve injuries which cause the disruption of all nerve fibers and support-ing structures (endoneurium and perineurium) but the epineurium. Although nerve continuity is maintained, and thus regeneration can occur spontaneously, nerve recovery might be poor due to scar tis-sue formation and mis-orientation of regenerated axons. Finally, in fifth-degree injuries, complete nerve transection occurs leading to the impossibility to get axonal regeneration unless nerve continuity is reconstructed surgically. In 1988, MacKinnon and Dellon pro-posed a sixth-degree to Sunderland's original classification which refers to complex nerve lesions where a combination of different degrees of injury takes place.

After injury, morphological changes occur not only at the injury site but also proximally and distally to it. Changes occurring in the soma of both motor and sensory neurons are related to a switch from a "signalling mode" to a "growing mode" (Fu and Gordon, 1997) with important changes in the synthesis of various proteins such as growth-associated proteins (Schreyer and Skene, 1991; Tetzlaff et al., 1991), cytoskeletal proteins (Fornaro et al., 2008) and neuropeptides (Hökfelt et al., 1994).

The proximal tract of the transacted nerve fibers shows a partial retrograde axon degeneration over few internodal segments with formation of bands of Büngner similar to those detectable in the distal nerve stump (Cajal, 1928). After an initial delay of few hours (Sunderland, 1978), a rich terminal sprouting occurs from the tip of proximal axon stumps. The regenerating sprouts grow up along the proximal bands of Büngner, across the injury site and finally along the distal bands of Büngner (Mira, 1984; Fawcett and Keynes, 1990; Witzel and Brushart, 2003). In the distal nerve segment a peculiar process, known as Wallerian degeneration (Stoll and Müller, 1999), starts immediately after injury. Wallerian degeneration is charac-terized by myelin breakdown, proliferation of Schwann cells and recruitment of macrophages. Disintegration of axoplasmic micro-tubules and neurofilaments by proteolysis starts within the first few days (Vial, 1958; Schlaepfer, 1977; Lubinska,´ 1982). The loss of axon–Schwann cell contact is a signal that induces proliferation of Schwann cells and upregulation of several types of neurotrophic factors, such as NGF (Heumann, 1987; Thoenen et al., 1988), BDNF, NT-3, NT-4/5, and NT-6 (Funakoshi et al., 1993), and the glial growth factor neuregulin (Audisio et al., 2008).

One of the most peculiar morphological changes in Wallerian degeneration is the formation of columns of Schwann cells (named bands of Büngner) which guide the regenerating axons with their basal membranes and neurite outgrowth-promoting factors, such as laminin and fibronectin (Baron-Van Evercooren et al., 1982; Liu, 1996; Hall, 1997). It has been shown that an excess number of axonal sprouts regenerate along the bands of Büngner (Sanders and Young, 1946; Aguayo et al., 1973) and thus their initial number detectable in the distal nerve segment usually exceed the number in the proximal nerve segment (Povlsen and Hildebrand, 1993). With time, a pruning process takes place and only regenerated axons that have reached a proper distal target survive (Sanders and Young, 1946), while the other axons degenerate (Griffin and Hoffman, 1993). Yet, a morphological change typical of regenerated nerves that was already pointed out by Cajal (1928), is "compart-mentation" (Morris et al., 1972, Lundborg, 2004), i.e. the formation of "minifascicles" in the distal nerve stump which replace the orig-inal large fascicles and make it possible to recognize a regenerated nerve even long time after injury.

3. Tissue engineering of peripheral nerves

It is beyond the aims of this paper to address the issue of the clinical indications of nerve surgery. Clearly, severe neurotmesis lesions with interruption of epineurial continuity require surgical reconstruction which is usually represented by direct nerve suture of the two stumps (end-to-end neurorrhaphy). When a nerve defect occurs, direct “tension” suture is harmful (Yi and Dahlin, 2010) and a guide must be used to bridge the gap (Millesi, 1970). The nerve guide is usually taken from the same patient’s sural nerve. Although autologous grafting causes a slight donor site mobility, it must be clearly pointed out that, today, it is still the gold stan-dard of nerve gap reconstruction in the clinics (Battiston et al., 2009a,b; Siemionow and Brzezicki, 2009). However, in order to avoid donor site morbility, an artificial nerve guide could be used to bridge the gap, especially if the gap is small and in sensory nerves; this approach is usually referred to as tubulization (Battiston et al., 2005; Geuna et al., 2007). Yet, peripheral nerve surgeons are also very interested in the development of artificial nerve grafts due to the fact that in severe and/or multiple nerve lesions (like in brachial plexus injury) there is often a limit of donor nerves. Unfortunately, up to now, except for simple tubular implants (Kehoe et al., in press), no bioengineered artificial nerve has been used for supporting axon elongation in order to bridge critical peripheral nerve defects in humans.

The first attempts to reconstruct peripheral nerves were already described by Galen in the second century A.D. (Terzis et al., 1997; Naff and Ecklund, 2001; Battiston et al., 2009a) and were followed, over the centuries, by other sporadic descriptions of nerve sutures such as those reported by Paul von Aegina in the seventh century (Streppel et al., 2000), Rahzes and Avicenna in the ninth century (Sunderland, 1981), Guglielmo di Saliceto, Guido Lanfranchi, Guy de Chauliac, Leonardo di Bertapaglia (Ladenheim, 1989; Terzis et al., 1997), and Gabriele Ferrara (cited in Artico et al., 1996) who was the first to provide a comprehensive description of nerve the suture procedure. Along the second part of nineteen century, based on the milestone basic science observations of Augustus Waller (1850) (reprinted in Stoll et al., 2002), nerve reconstruction saw major advancements leading to the first attempts of nerve tissue engineering. Philipeaux and Vulpian (1870) and Albert, 1885 were the first to describe the repair of nerve defects by means of autologous nerve segments. Few years later, Neuber (1879), Glück (1880) and Vanlair (1882), described a method for nerve repair based on the employment of another tissue (biological tubulization), namely a piece of bone. Biological tubulization has received much attention over the last century and the best results have been obtained using veins and skeletal muscle tissue (Chiu and Strauch, 1990; Pereira et al., 1991; Geuna et al., 2004; Tos et al., 2007). Also the use of a non biological conduit for nerve repair (synthetic tubulization), a strat-egy that was first attempted by Payr already in 1900, has recently seen a tremendous development due to the potential commercial spin-off of biomaterials for clinical applications (Luis et al., 2007; Siemionow et al., 2010).

Over recent years, transplantation is acquiring more and more importance in surgery, moving from whole organ transplants to transplantation of only parts of an organ (tissues and cells), thus

expanding the employment of autotransplantation which avoids the problems related to rejection. In particular, for peripheral nerve reconstruction a great attention among researchers has been attracted by autologous cell transplantation therapies (Tohill and Terenghi, 2004; Pfister et al., 2007). Of course, the choice of the cell type to be used for transplantation is the key factor for the therapeutic success and various types of cells have been shown to promote axonal regeneration after their transplantation inside nerve conduits (Siemionow and Brzezicki, 2009). One of the most promising approaches is represented by glial cell transplantation since these cells are known to play a major role in axonal regen-eration by secreting neurotrophic factors and participating in the myelinic and amyelinic ensheathing of regenerated axons (Radtke and Vogt, 2009). Two types of gial cells have received much atten-tion so far: Schwann cells (SCs), which represent the glia of most peripheral nerves, and olfactory ensheathing cells (OECs), which are the glia of the olfactory nerve.

SCs, besides ensheathing peripheral nerve axons, also secrete growth factors considered essential for the survival of the neuronal cells and the promotion of the regeneration process (Hall, 2001). After peripheral nerve injury, SCs together with macrophages, remove necrotic tissue and myelin debris. Furthermore, SCs pro-liferate to form Büngner bands, which help the regrowing axons to elongate their growth cones in the direction of denervated targets (Geuna et al., 2009). During nerve regeneration SCs create a suit-able environment for axonal growth by expressing cell-adhesion molecules and forming an endoneurial sheath that acts as a guide for regenerating axons. Because of their crucial involvement in the regeneration of injured peripheral nerves, the attempts to use SCs to enrich the nerve conduits are continuously increasing. Hadlock et al. (2000) and Mosahebi et al. (2001) showed, in the rat, that SC transplantation inside different types of nerve conduit leads to the improvement of both quality and rate of axon regeneration. SC-seeded vein conduits proved also to be effective, in the rabbit, in bridging long nerve defects up to 4 cm (Zhang et al., 2002) and 6 cm (Strauch et al., 2001), whereas the use of the vein conduit alone in the same experimental conditions was ineffective.

Recently Goto et al. (2010) studied in vitro the efficiency of a new approach for the repair of the injured peripheral nerve based on cultures of SCs on a rolled sheet of collagen gel and showed that this construct kept the viability of SCs and promoted axonal outgrowth.

Whereas several experimental animal studies showed the effec-tiveness of SC transplantation to promote nerve regeneration, Mosahebi et al. (2001) pointed out that the use of autologous cul-tured SCs may be impractical for the treatment of acute nerve injuries in the clinics because of the time required for harvest-ing and expanding SCs; actually, up to 10 weeks of SC culture may be required to get a sufficient amount of cells. Yet, isola-tion of SCs is complicated because of the frequent contamination of fibroblasts (Mosahebi et al., 2001). In order to cope with the latter problem, Wei et al. (2009) adopted a new purification tech-nique of rat SCs which is based on the removal of contaminating fibroblasts by means of a combination of Ara-C (cytosine-B arabi-noside hydrochloride) and a differential cell detachment technique, a method which may give rise to a stable SC yield with a final purity above 99.2% within 10 days.

Recently, olfactory ensheathing cells (OECs) withdrawn from the olfactory nerve have raised great interest for neural repair purposes because of their homing capacity in both peripheral and central nervous system. OECs, which guide the continuously regenerating axons of the olfactory neuroepithelium towards the olfactory bulb, have shown to retain a higher migratory potential and ability to penetrate glial scars in comparison to SCs (Franklin and Barnett, 1997), a property which makes them a rational trans-plantation candidate for nerve reconstruction. The employment
of OEC transplantation in injured peripheral nerves have shown that, in rodents, these cells can provide trophic support, form cel-lular bridges across the site of injury, and significantly promote axonal regeneration (Verdú et al., 1999; Guntinas-Lichius et al., 2001; Radtke et al., 2005, 2010; Dombrowski et al., 2006).

Transplantation of OECs (Radtke et al., 2005; Dombrowski et al., 2006) into the regenerating sciatic nerve of rodents showed that OECs survived, distributed longitudinally across the lesion site and were integrated into the repaired nerves, contributing to the myelin formation of regenerated axons. These authors hypothe-sized that, after lesion, transplanted OECs are primed to produce neurotrophins and, therefore, can have an immediate effect on the injured axons before scar formation occurs. Thus, it can be hypothetically tenable that OEC transplantation at the time of microsurgical repair of peripheral nerves may provide a scaffold for axons to regenerate as well as trophic support and directional cues leading to increased axonal regeneration across the repair site and improved functional outcome (Radtke and Vogt, 2009).

So far, OECs have been mainly obtained from primary cultures. Like all primary cell cultures, their preparation has various dis-advantages, such as labor-intensive operator workload, small cell amount, other cell-type contamination (especially fibroblasts and astrocytes) and limited survival in culture (DeLucia et al., 2003; Moreno-Flores et al., 2006). In alternative to primary glial cell cultures, immortalized clonal cell lines have been proposed for experimental animal studies (Lakatos et al., 2000; DeLucia et al., 2003).

Goodman et al. (1993) described an immortalized cell line
– named Neonatal Olfactory Bulb Ensheathing Cell (NOBEC) – that was derived from neonatal OECs by transduction with SV40 large T cell antigen. It has been shown that NOBECs, though immor-talized, are minimally transformed, maintain viable monolayers without forming tumors when transplanted into rats and have a low proliferation rate in comparison to glioma cells (Goodman et al., 1993). NOBECs produce the same growth-promoting proteins as primary OEC cultures (Goodman et al., 1993), possess regeneration-promoting capabilities, and retain the major glial features (Audisio et al., 2009).

Since the relatively small amount of glial cells that can be obtained from primary cultures is considered a limitation for clini-cal applications (Mosahebi et al., 2001), the use of stem cells that can be differentiated in glial cells in vitro before transplantation, have also been investigated. Neural stem cells (NSCs) have been tested for nerve regeneration purposes in animal models since they have the potential to differentiate into both neurons and glia (Bithell and Williams, 2005).

Murakami et al. (2003) have used a silicone tube enriched with transplanted NSCs for repairing rat nerve defects and found that this approach promote axonal regeneration. Transplantation of NSCs were also found to promote axonal regeneration after chronical transection of rat nerves (Heine et al., 2004).

Although the use of undifferentiated NSCs has led to good exper-imental results, it should be noted that tumor formation following NSC transplantation in a rat peripheral nerve injury model was reported thus pointing to the importance of comprehensive in vitro characterization of cells prior to transplantation for avoiding sub-clones which may become tumorogenic (Radtke et al., 2010). Yet, it should also be noted that in vitro NSC pre-differentiation before conduit enrichment did not lead to positive results in a rat neu-rotmesis experimental model (Amado et al., 2008; Luís et al., 2008; Amado et al., 2010).

In alternative to NSCs, mesenchymal stem cells (MSCs) have been widely investigated for improving peripheral nerve regen-eration. They can be easily obtained, purified and expanded in culture and offer a potentially unlimited source of cells for tissue engineering (Caplan and Dennis, 2006). They can be derived from various stem cell niches in adult tissues by means of minimally invasive approaches. Although bone marrow is the most charac-terized source of MSCs, numerous reports have demonstrated that MSCs can also be isolated from adipose tissue, fresh or banked human umbilical cord blood, and tooth pulp (Alhadlaq and Mao, 2004).

Advances in stem cell biology and manipulation (Tohill and Terenghi, 2004) have opened new perspectives since MSCs are thought to be able to differentiate into multiple cell lineages, such as osteoblasts, chondrocytes, myoblasts, adipocytes, neuron-like cells and glial-like cells (Alhadlaq and Mao, 2004; Raimondo et al., 2006; Mantovani et al., 2010). Yet, MSC capability of self-replication to many passages makes them potentially expandable to sufficient numbers for allowing regeneration of large tissue defects.

Recently, Cho et al. (2010) showed that human MSCs can be differentiated into neural cells in vitro and, when transplanted in the injured facial nerve of the guinea pig, they were effective in promoting nerve regeneration. Moreover it has been shown that also SCs can be derived from rat MSCs (Kingham et al., 2007). It has been shown that MSCs can differentiate into Schwann cell-like phenotype, with the morphological, molecular and functional char-acteristics of regenerative SCs, both in rat (Caddick et al., 2006) and in human (Brohlin et al., 2009) thus opening interesting per-spectives in the clinical view since this approach may facilitate the availability of adequate quantity of autologous SCs from the same patient. It has also been shown that rat adipose-derived MSCs have potential to myelinate during regeneration (Mantovani et al., 2010).

Cell transplantation however, is not the only pillar of tissue engi-neering and local delivery of growth factors and other molecules which can promote tissue repair is also emerging as one of the key issues in regenerative medicine. One of the most promising strategies for local growth factor delivery is probably

gene trans-fer and this approach is also getting more and more interest in the repair of the peripheral nervous system (Haastert and Grothe, 2007; Tannemaat et al., 2009; Zacchigna and Giacca, 2009; Pereira Lopes et al., in press). Various studies in animal models have shown that peripheral nerve regeneration can be improved by gene trans-fer. For instance, it has been demonstrated that over-expression of FGF-2 by transplanted SCs can improve length and number of regenerating axons after rat peripheral nerve repair (Timmer et al., 2003). Similar results were obtained by Haastert et al. (2006) who focused on the different effects of rat Schwann cell gene trans-fer with low and high molecular weight FGF-2 isoforms and find out that 18-kDa-FGF-2 mediated inhibitory effects on regenerating axons while 21-/23-kDa-FGF-2 induced early functional recovery and stimulation of myelination.

4. Improving regeneration after nerve reconstruction

Tissue engineering of peripheral nerves not only relies on the development of innovative microsurgical techniques and/or trans-plantation strategies and devices, but also on the combined use of other therapeutic tools which can improve the effectiveness of nerve reconstruction.

First, the possibility to manipulate the regeneration process by means of drug administration before, during and/or after nerve repair should be considered (Magnaghi et al., 2009). Unfortunately, in spite of the several experimental animal studies that showed the effectiveness of a number of molecules, such as hormones and drugs, for promoting nerve regeneration, there is no established treatment protocol for promoting nerve recovery after surgical reconstruction.

Second, the possibility to improve the effectiveness of periph-eral nerve tissue engineering by means of physical therapy should also be taken into great account since many studies have shown that various physical agents applied during and after nerve recon-struction can significantly increase functional recovery. The most promising approaches, already tested in animal models, include electrical stimulation (Panetsos et al., 2008), manual stimulation (Bischoff et al., 2009), and photo-stimulation (Rochkind et al., 2009).

Finally, a particular mention deserves the need to investi-gate brain plasticity related to nerve tissue engineering, i.e. the adaptation changes occurring in the central nervous system as a consequence of peripheral nerve repair and regeneration (Navarro, 2009; Herrera-Rincon et al., 2010). While it is widely acknowl-edged that changes occurring after peripheral nerve injury and regeneration induce changes to the central motor and sensory pathways, unfortunately the large majority of nerve repair and regeneration studies in experimental animal models are exclu-sively based on the investigation of what happens at the lesion site and/or at the level of the distal nerve trunk. Central changes can occur at various cortical and sub-cortical stations along motor and sensory pathways and it can be foreseen that a better knowl-edge about brain plasticity mechanisms related to peripheral nerve tissue engineering might represent the basis for directing the development and validation of new and more effective treatment strategies.

5. The importance of publishing negative results in nerve tissue engineering

A very important issue in nerve tissue engineering is the pub-lication of negative results. Positive result bias is a well-known phenomenon in scientific literature (Hasenboehler et al., 2007) the main reasons to be found in the unwillingness of researchers to publish negative results which are often, though erroneously, per-ceived as a scientific failure.

In peripheral nerve regeneration studies, this occurrence might be even more pronounced for a couple of other reasons. First, the involvement of industrial companies, due to the frequent employment of biomaterials and/or stimulation devices, which might be unwilling of publishing a study that prospects a null or even negative effect of one of their products. Second, and perhaps most important, nerve regeneration studies are based experiments which take long time to be completed. Postoperative observa-tion may last

for several months and thus results of one single experiment may require more than one year or more to get the preliminary results. When additional experiments are required (as it usually occurs) study's duration may last very long and thus, if indications of the ineffectiveness (or negative effects) of a new therapeutic approach arise from the first experiments, researchers might decide to give up with the study rather than try to complete it and publish its results. However, it should be clearly pointed out that divulgation of negative results is very important since it avoids repetition of unsuccessful experiments and facilitate the concen-tration of the research resources on the most promising approaches for improving repair and regeneration of injured peripheral nerves.

Finally, researchers, especially the youngest ones, might believe that there is a lack of interest among editors to accept in their jour-nals papers which report on negative results. Actually, this does not appear to be the case in the nerve regeneration research and, espe-cially in recent years, several papers have been published reporting negative results after various tissue engineering approaches in experimental animal models (Amado et al., 2008; Grosheva et al., 2008; Haastert et al., 2009; Sinis et al., 2008, 2009; Skouras et al., 2009).

6. Conclusions

Unlike the adult central nervous system, where the potential for axonal growth and neurogenesis is very limited and plasticity (in response to stimulus) and regeneration (in response to injury) are mainly represented by adaptive changes in synaptic reorga-nization and neural circuitries, plasticity and regeneration in the adult peripheral nervous system are more pronounced and they are predominantly based on axonal re-growth and neuron addi-tion (Geuna et al., 2010). However, especially in case of severe nerve lesions with substance loss, regeneration and functional recovery are usually partial and often frankly unsatisfactory (Höke, 2006; Lundborg, 2002; Midha, 2006; Millesi, 2006; Ruijs et al., 2005; Samii et al., 1997; Casha et al., 2008; Battiston et al., 2009b) thus calling for more research which should strive for a new level of innovation which will bring together various different disciplines from basic to clinical. The recent scientific advances have clearly pointed out that peripheral nerve repair and regeneration cannot be any more a mat-ter of surgical reconstruction only, but should rather be addressed from multiple and interdisciplinary viewpoints (Battiston et al., 2009a,b). To say it in other words: In peripheral nerve injury, perfect microsurgical repair is just part of the story!

In this review we have synthetically overviewed a huge body of literature which touches several scientific disciplines that are very different among them. The key discipline is of course recon-structive microsurgery and, in order to strengthen the translational approach, we believe that it is very important to emphasize that clinical scientists should be whenever possible involved not only at the end of the research (when basic science results appear to be ready to be tested for a clinical application) but also in the very early research steps (when basic science experiments are designed). In this way, clinicians can follow up the research in all its phases and will eventually be more motivated in applying its results with patients.

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